Glutathione reductase and 6-phosphogluconic dehydrogenase in hæmolysates, unlike glucose-6-phosphate dehydrogenase, remain active even after incubation with stromata or 'Norit'. This allows preliminary investigation of the binding of triphosphopyridine nucleotide to glucose-6-phosphate dehydrogenase because the former is also the coenzyme of 6-phosphogluconic dehydrogenase. Stroma-free dialysed hæmolysates retain sufficient triphosphopyridine nucleotide for the hæmolysate to reduce GSSG in 1 hr. at 37° C. when 6-phosphogluconate, but not triphosphopyridine nucleotide, is added to the reaction mixture. However, under the same conditions, GSSG is not reduced after incubation with stromata or 'Norit'. (Triphosphopyridine nucleotide is retained in hæmolysate which has been dialysed for as long as 42 hr.)

The inactivation of glucose-6-phosphate dehydrogenase and loss of triphosphopyridine nucleotide remaining in dialysed hæmolysates occur together, suggesting that the non-dialysed fraction of triphosphopyridine nucleotide is bound to glucose-6-phosphate dehydrogenase and that this is active only when bound with its coenzyme. The 6-phosphogluconic dehydrogenase does not bind triphosphopyridine nucleotide or require it for stabilization.

Intact human erythrocytes have pyridine nucleotidase activity, both on the surface and within the cell: the surface activity is demonstrated by the inactivation of glucose-6-phosphate dehydrogenase in isotonic hæmolysates during incubation with whole human erythrocytes; the intracellular effect is shown by a loss of glucose-6-phosphate dehydrogenase activity and bound triphosphopyridine nucleotide in erythrocytes incubated in isotonic saline for 2 hr. at 45° C. Although no loss of glucose-6-phosphate dehydrogenase activity occurred during storage of non-sensitive blood, gradual loss of glucose-6-phosphate dehydrogenase activity in sensitive blood during four weeks of storage in acid–citrate–dextrose solution also suggests an intracellular action of pyridine nucleotidase.

These results show that triphosphopyridine nucleotide stabilizes glucose-6-phosphate dehydrogenase in hæmolysates as well as in partially purified preparations and confirm the suggestion that this stabilization may be related to the inactivation of this enzyme by erythrocytic stroma (ref. 5 and previous communication). Partial stabilization of glucose-6-phosphate dehydrogenase by nicotinamide is indirect, by protection of the pyridine nucleotides from pyridine nucleotidase activity of stromata. Stabilization by diphosphopyridine nucleotide may also be indirect, by its enzymatic conversion to triphosphopyridine nucleotide, since diphosphopyridine nucleotide does not stabilize glucose-6-phosphate dehydrogenase of partially purified preparations[5]. Nevertheless, protection of glucose-6-phosphate dehydrogenase of stroma-free hæmolysates by di- as well as triphosphopyridine nucleotide indicates that the former can help to stabilize glucose-6-phosphate dehydrogenase in human erythrocytes, even though it is not a coenzyme for it. The key to the mechanism of primaquine hæmolysis, and possibly of cellular ageing, may be related to these stabilizing processes.

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PAUL E. CARSON
STANLEY L. SCHRIER
ROBERT W. KELLERMEYER

Department of Medicine,
University of Chicago,
Chicago, Illinois.

[1] Carson, P. E., Flanagan, C. L., Ickes, C. E., and Alving, A. S., *Science*, **124**, 484 (1956).
[2] Carson, P. E., Schrier, S. L., and Alving, A. S., *J. Lab. and Clin. Med.*, **48**, 794 (1956).
[3] Kirkman, H. N., *Fed. Proc.*, **18**, 261 (1959).
[4] Feigelson, P., Williams, jun., J. N., and Elvehjem, C. A., *J. Biol. Chem.*, **185**, 741 (1950).
[5] Kirkman, H. N., in Symposium on Molecular Genetics and Human Disease (in the press).

X-RAY DAMAGE AND RECOVERY IN MAMMALIAN CELLS IN CULTURE

By M. M. ELKIND and HARRIET SUTTON
National Institutes of Health, U.S. Public Health Service, Bethesda 14, Maryland

As measured by the ability to sustain unlimited proliferation, the X-ray sensitivity of microorganisms has been generally observed to be much greater than the sensitivity of important macromolecules. This, in itself, has been an important reason for associating the lethal effect of X-rays with the genetic apparatus of the cell. Puck and Marcus's[1] observation that mammalian cells in tissue culture were even more sensitive—10–100 times or more—than bacteria or yeasts further reinforced this view and led to their very reasonable proposal that the sensitive sites in mammalian cells are the chromosomes.

If the functional integrity of the genetic apparatus is required for viability, since the survival curves of most somatic cells are sigmoid (or multihit), it might be expected that survivors after X-irradiation would be more sensitive to subsequent exposure than the parental population. This follows from the fact that multihitness implies a threshold type of response (which means damage must be accumulated before an effect is observed) and hence that surviving cells accumulated a sublethal amount of damage.

We have investigated the question of the presence of heritable damage in two cell lines of the Chinese hamster, *Cricetulus griseus*[2] (clone *A* ovarian tissue and strain *V* female lung tissue), propagated in tissue culture. We have found that essentially all the survivors after X-irradiation did not display heritable damage, as would be evidenced by their radiosensitivity, but rather that they repaired their accumulated damage before their first division after irradiation.

Our growth medium bears the designation *HU*-15. It consists of Eagles's amino-acids and vitamins[3] with glutamine at a concentration of 1 m*M*; 4 per

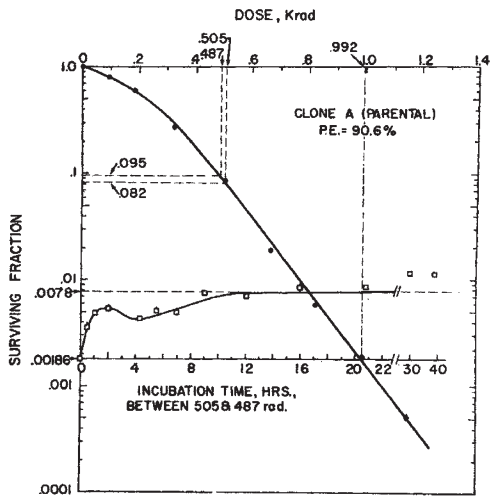

Fig. 1. Recovery of X-irradiated cells (between 505 rad and 487 rad doses) as a function of time of incubation at 37° C.

cent Earle's *NCTC*-109 [4]; Puck's[5] saline *F* with the calcium content increased 6·5 times; and 15 per cent undialysed fœtal calf serum (Colorado Serum Co., Denver). Both cell lines grow in this medium with a doubling time of about 12 hr.

Our X-ray source consisted of a Machlett *OEG*-60 tube powered by a full-wave rectified, 55 kV. supply. The tube was operated at 12 m.amp. with 0·175 mm. aluminium filtration; absorbed dose-rate 720 rads/min. After the attachment of cells in 9-cm. Petri dishes, the plate covers and growth medium were removed and the cells were irradiated at room temperature in a humidified atmosphere of 2 per cent carbon dioxide in air. Surviving clones were stained and counted after 12–18 days incubation at 37° C. in a 2 per cent carbon dioxide incubator. Identification of abortive colonies was facilitated by the use of a projection technique, although our results are essentially the same whether or not abortive colonies are included. Plating efficiencies in most of our experiments were about 70 per cent; essentially the same results were obtained, however, in experiments having plating efficiencies from 10 to 90 per cent.

In discussing the observations, a multihit model will be assumed for simplicity although our conclusions apply equally as well to sigmoid or threshold type survival curves in general. For lag-phase cells, clone *A* was found to display hitness numbers of 4–5 and clone *V* 6–7.

X-ray dose fractionation was employed to test for repair of accumulated damage. Fig. 1 shows a survival curve for single clone *A* cells trypsonized and plated 2 hr. before exposure. (Standard errors are indicated where larger than the plotted points.) In addition, the lower portion of the figure shows a recovery curve for cells which had received a first dose, 505 rads, followed by incubation at 37° C. for various periods of time before receiving a second dose of 487 rads.

If there had been no recovery between the exposures, the two doses would have been completely additive, and the survival after a total of 992 rads would have been 0·0019. Alternatively, if there had been complete recovery between doses, the survival to the first and second doses would have been 0·082 and 0·095, respectively. The product of these latter values is 0·0078, which represents the survival corresponding to complete recovery between doses. The points to be noted are: (1) as a function of time at 37° C., the cells recover in a manner which may involve repair of sites as well as fluctuations in sensitivity; (2) for clone *A* cells, recovery appears to be complete by about 10 hr. and constant until about 25 hr.; and (3) the survival rises above 0·0078 after about 25 hr., which probably represents the effect of cellular multiplicity on survival concomitant with the onset of post-irradiation division.

The likelihood of the last point was arrived at by two types of measurements. First, we showed that the 'principle of cellular multiplicity' holds for these cells. That is, the surviving fraction of colonies containing more than one cell is shifted upward by an amount governed by the average cellular multiplicity providing that each cell in the colony has the same average sensitivity and that it must be inactivated independently to suppress post-irradiation colony formation. Secondly, an estimate of division delay following a first dose of 505 rads was made by comparing the clonal growth of irradiated cells with unirradiated controls. The comparison was made after clones had reached a size, about 100 cells per clone, which permitted an unambiguous identification of such clones as survivors. In agreement with the recovery curve in Fig. 1, the latter measurement indicated a division delay of about 30 hr.

In addition to the preceding, we have also shown that recovery can take place at room temperature unaccompanied by division in the control population.

To verify that the plateau region in the recovery curve of Fig. 1 represents complete repair of the accumulated damage resulting from the first dose, the survival curve was repeated after 505 rads followed by 18·1 hr. at 37° C. In Fig. 2, the non-fractionated survival curve was re-drawn starting from the survival corresponding to a first dose of 505 rads. The figure displays an excellent fit of the re-drawn curve to the observed points and shows that, in the exponential regions of both curves, maximum recovery shifts the survival upward by a factor equal to the hitness number. It also follows from Fig. 2 that, at least in the region of full recovery, synergism between the first and second doses is probably absent and therefore the second dose measures the degree of repair of damage resulting from the first dose.

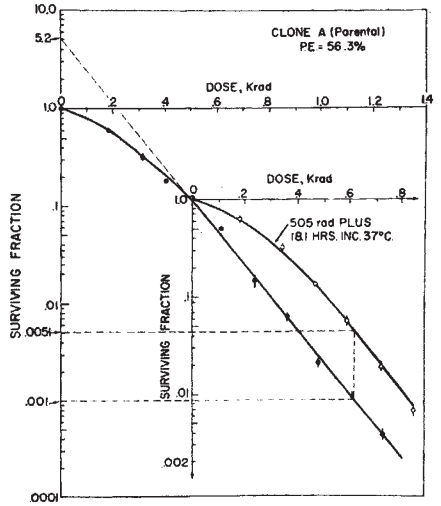

Fig. 2. Full recovery after a dose of 505 rads followed by 18·1 hr. at 37° C. as evidenced by the repetition of the non-fractionated survival curve

The preceding figures are part of a comprehensive study which will be reported in detail elsewhere. To the extent that these and the results to be reported are typical for somatic cells in general, it should be noted that: (1) The vast majority of surviving cells completely repair their accumulated damage before their first division post-irradiation. This means that if the hitness number is n, in the exponential region, survivors undo the effects of a maximum number of hits which is $n-1$. (2) The kinetics of recovery depend on the physiological state of the cells and/or can be caused to appear to undergo large oscillations depending on the recovery medium. These apparent oscillations may result from the combined effects of changes in sensitivity and repair of inactivated sites. (3) Although there are important quantitative differences, log-phase cells respond similarly. (4) A cell can undergo repeated cycles of damage and repair with no apparent attenuation of the repair process(es).

There are several contexts in which these findings are of interest. If the chromosomes are the X-ray sensitive sites and chromosome breaks are the hits leading to lethality, then some new properties of restitution must be considered. First, restitution goes to completion in surviving cells. Secondly, the cell's ability to restitute breaks remains unimpaired after repeated doses. In view of the preceding, Puck's[6] report of a high yield of mutant characteristics in the progeny of cells surviving 5–7 mean lethal doses may be applicable to the material he was using; may be evidence of a radiation-induced chromosomal lability which is expressed after recovery and during clonal growth; may imply that mutation production and lethality are not, in general, closely connected; or may indicate that the chromosomes are not the primary sensitive sites related to viability.

Another area in which these results may apply is in connexion with tumour therapy. Treatment protocols involving fractionation are common, permitting, in general, ample time between treatments for considerable if not complete recovery. Even for situations in which the hitness number may only be 2, a simple calculation reveals that, if recovery is not duly accounted for, the survival using fractionation can be higher than expected by several orders of magnitude. Of course, tissue recovery in a general sense has been recognized by radiation therapists for a long time. These results, however, provide a cellular basis for this phenomenon and lend specific direction to the research that should be undertaken both to take advantage of, as well as to control, this effect.

Additional experiments are planned (or in progress) to examine the influence of dose-rate on survival, and the biochemical and cytogenetic aspects of recovery.

We are indebted to Dr. T. T. Puck for a sample of his clone *A*, ovarian tissue, which has been propagated in our laboratory without recloning since August 1958; to Dr. Denys Ford for his *V* strain, female lung tissue, which was recloned in December 1958; and to Dr. George Yerganian, who supplied the Chinese hamsters for the original explants.

[1] Puck, T. T., and Marcus, P. I., *J. Exp. Med.*, **103**, 653 (1956).
[2] Yerganian, G., *J. Nat. Cancer Inst.*, **20**, 705 (1958).
[3] Eagle, H., *Science*, **122**, 501 (1955).
[4] Evans, V. J., Bryant, J. R., McQuilkin, W. T., Fioramonti, M. C., Sanford, K. K., Westfall, B. B., and Earle, W. R., *Cancer Res.*, **16**, 87 (1956).
[5] Puck, T. T., Cieciura, S. J., and Robinson, A., *J. Exp. Med.*, **108**, 945 (1958).
[6] Puck, T. T., *Proc. U.S. Nat. Acad. Sci.*, **44**, 772 (1958).

TALLOWY DISCOLORATION IN CHEDDAR CHEESE

By STAFF OF THE WALLACEVILLE DAIRY LABORATORY
Wellington, New Zealand

Fat Oxidation and Trace Metals in Cheese

A SERIOUS fault in mature Cheddar cheese is the gradual appearance of bleached areas with a tallowy flavour, aptly described as 'tallowy discoloration' or 'white streak'. The erratic incidence of this defect has for long hindered efforts to find the prime cause. In seeking the cause, most of the chemical features examined showed little, if any, difference between normal and tallowy portions, apart from the oxidized condition of the latter. The most striking differences were found in the disposition of trace metals. As compared with adjacent normal cheese the copper content of the centre of tallowy portions was always much lower, often as little as a third; but the iron content was always higher, usually by about a half. Experiments with threefold added copper did not affect the incidence of the tallowy defect, nor did added iron salts.

When normal cheese curd was treated with an excess of warm 5 per cent iron-free brine, the portion that dissolved contained more iron than the undissolved cheese, which was shown to lose a corresponding quantity of iron. Moreover, treatment for a shorter period so that less cheese dissolved, resulted in a higher iron content of the dissolved cheese. This indicated the presence of an iron compound more soluble than the cheese, and also suggested a probable connexion between the salting process in cheese-making and the appearance of tallowiness.

The degree of oxidation of iron in cheese was also studied. Practically no ferrous iron could be extracted from young cheese; but the amount extractable increased with ageing at variable rates, some cheese yielding very little at maturity. Cheese tending to tallowiness had increased ferrous iron content, while the actual tallowy seams had the highest ferrous content. This occurred in seams where oxidation was most advanced, as shown by high peroxide values and also by oxidation of the sulphydryl groups of the protein. These results now indicate that an important part is played by an iron complex. The very low copper content at the centre of tallowy seams may be an effect of oxidation of a copper–sulphydryl compound similar to that demonstrated by Stricks and Kolthoff[1].

Most of these investigations were carried out during a period of several years. In view of the recent finding by Rammell (following communication) that hæmatin compounds can cause tallowy discoloration, they support the view that these or similar iron complexes